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(54) Title: COMPOSITION AND METHODS FOR CREATING SYNGENEIC RECOMBINANT VIRUS-PRODUCING CELLS

(57) Abstract

Replication-defective viruses and means for intracellular replication thereof are described which are useful for gene therapy. Human cells can be changed into recombinant replication-defective virus particle-producing cells by the simultaneous delivery to those cells of two different nucleic acids: the first being a replication-defective viral genome, the second being a nucleic acid that complements the viral sequences deleted from the first nucleic acid so as to result in the production of new infective virus. The first nucleic acid can be delivered by the replication-defective virus itself or, as a nucleic acid that is not part of the virus. In a preferred embodiment, the replication-defective virus includes elements to maintain the two nucleic acids in combination during transduction. Examples of preferred viral sources are adenoviruses, herpesvirus, retroviruses, and adeno-associated viruses. Nucleic acids useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins that will serve to treat a pathophysiologic condition within the body.

1

COMPOSITION AND METHODS FOR CREATING SYNGENEIC RECOMBINANT VIRUS-PRODUCING CELLS

Background of the Invention

5   The present invention is in the general field of gene therapy, and is in particular in the area of engineering viral vectors for transduction of normal cells.

"Gene therapy" refers to the treatment of pathologic conditions by the addition of exogenous

10   nucleic acids to appropriate cells within the organism. Nucleic acids must be added to the cell, or transduced, such that they remain functional within the cell. For most gene therapy strategies, the new nucleic acids are designed to function as

15   new genes, i.e., code for new messenger RNA that in turn codes for new protein. As originally conceived, gene therapy was directed towards monogenetic disorders like adenosine deaminase deficiency and cystic fibrosis. It has become

20   abundantly clear that gene therapy might also be helpful in polygenetic somatic disorders like cancer.

The rapid implementation of gene therapy in human trials has been made possible by the

25   development of relatively efficient means of adding new nucleic acids to cells, a process generally referred to as "gene transduction". The clinically applicable gene transduction methods fall into one of three categories: a) cationic lipids (b)

30   molecular conjugates (c) recombinant viruses. These different means of accomplishing gene transduction have been recently reviewed (Morgan 1993 Ann. Rev. Biochem. 62:191.; Mulligan 1993 Science 260:926; Tolstoshev 1993 Ann. Rev. Pharm.

35   Toxicol. 32:573).

Although the three major groups of gene transduction methodology are relatively efficient,

2

the percentage of target cells that can be
transduced *in vivo* remains relatively low. For the
treatment of conditions requiring a higher
percentage of gene transduction, new technologies
5   for increasing the percentage of transduced cells
would be a very useful advance.

One pathophysiologic condition where
transduction efficiency has been shown to be a
limiting factor is cancer. Some strategies for
10   cancer gene therapy entail the addition of toxin
genes, or other genes deleterious to cancer growth,
to the tumor mass. These approaches have been
facilitated by the use of toxin genes that kill not
only the transduced cells, but also adjacent cells
15   by a "bystander effect". The herpes simplex virus
thymidine kinase + ganciclovir system exemplifies
the desired bystander effect (Freeman 1992 Cancer
Research 53:5274). In this case, the viral
thymidine kinase gene converts the prodrug,
20   ganciclovir, into a phosphorylated nucleoside
analog that blocks DNA replication and thereby
further growth (Paul 1992 Amer. J. Med. Sci.
304:272). The phosphorylated ganciclovir bystander
effect has recently been shown to be the
25   consequence of gap junction - mediated transfer of
the toxin to adjacent cells (Stambrook 1993 Cancer
Gene Therapy 1(suppl):1).

However, the benefit of the bystander
effect in treating animal models of solid tumors
30   has required the administration of viral producing
cell lines to yield adequate transduction with the
consequence of measurable reductions in tumor mass.
This strategy was first reported by Culver et al
for the treatment of brain tumors engrafted in
35   nonhuman primates (Culver 1992 Science 256:1550).
In this report, a murine cell line that produced a
recombinant retrovirus containing a viral thymidine

3

kinase gene was administered to the engrafted tumor
mass, the concept being that the tumor cells would
be continually exposed to new virus over an
extended period of time. The animals were
5   subsequently treated with ganciclovir and
demonstrated dramatic reductions in tumor mass.
This concept has now been extended to liver tumors
and subcutaneously engrafted tumors (Caruso 1993
Proc. Natl. Acad. Sci. USA 90:7024; Freeman 1993
10   Cancer Res. 53:5274).

        The producer cell line approach does have
important limitations. First, the producer cells
are xenografts of immortalized cells that may be
quickly eliminated by host immune mechanisms.
15   Second, this method relies on the existence of
stable producer cell lines that are available only
for recombinant retroviruses, but not for other
recombinant viruses. The recombinant adenoviruses,
adeno-associated viruses, and herpes viruses are
20   all produced by lytic infections of their
corresponding "packaging" cell lines. Thus, these
viruses cannot be utilized for this type of cancer
therapy. Third, it has become apparent in the
first human trial of recombinant retroviral
25   producer cell treatment of brain cancers that
multiple injections were required to produce
partial responses in 5 of 8 patients treated (Ram
1993 Cancer Gene Therapy 1(suppl):1). This has
prompted these investigators to pursue a strategy
30   that utilizes multiple injections of the tumor mass
that is of unproven utility that will not be
applicable to other sites of tumor mass where the
tumor location cannot be as precisely fixed as is
possible within the rigid confines of the human
35   skull.

        The clinical applications of gene therapy
would be greatly advanced by the development of new

4

approaches to achieving high levels of gene
transduction *in vivo*. A particularly valuable
approach would be one that provides for the
continuous production of new recombinant vector
5   that is applicable to multiple vector systems and
does not require the administration of xenogeneic
cells.

It is therefore an object of the present
invention to provide vectors for gene therapy and
10   methods for use thereof not requiring xenogeneic
cells.

It is a further object of the present
invention to provide improved defective viral
vectors that can be co-transduced with elements for
15   intracellular reproduction of the defective viral
vectors, which are capable of infecting other
cells.

Summary of the Invention

Replication-defective viruses and means
20   for intracellular replication thereof are described
which are useful for gene therapy. Human cells can
be changed into recombinant virus-producing cells
by the simultaneous delivery to those cells of two
different nucleic acids: the first being a
25   replication-defective viral genome, the second
being a nucleic acid that complements the viral
sequences deleted from the first nucleic acid so as
to result in the production of new infective virus.
The first nucleic acid can be delivered by the
30   replication-defective virus itself, or as a nucleic
acid that is not part of the virus. In a preferred
embodiment, the replication-defective virus
includes elements to maintain the two nucleic acids
in combination during transduction.
35   Examples of preferred viral sources are
adenoviruses, herpesvirus, retroviruses, and adeno-

associated viruses. Nucleic acids useful for gene
therapy include those that code for proteins used
to identify cells infected with the recombinant
virus, those that encode for proteins that function
5   to kill cells containing the viral genome, or that
encode for therapeutic proteins that will serve to
treat a pathophysiologic condition within the body.
Commonly, the second nucleic acid sequence provides
sequences that in a trans configuration enable the
10   first nucleic acid to replicate and be packaged
into new, replication-defective viral particles
that can also contain other nucleic acids that are
useful for gene therapy. A preferred example of a
linking means for the two nucleic acids is to
15   conjugate on the surface of the defective virus
containing the first nucleic acid highly charged
polyamino acids, such as polylysine, which binds
ionically to the second nucleic acid.

Brief Description of the Drawings

20   Figures 1A and 1B are schematics of the
infection and replication processes of replication-
competent virus (Figure 1A) and replication-
incompetent, or defective, virus (Figure 1B).
           Figures 2A-2D are maps of the
25   complementary adenoviral nucleic acid sequences.
           Figure 2A is a map of the AdCMVlacZ viral
genome. Hatched bars represent the conserved human
type 5 adenovirus sequences with map units
indicated below. CMV promoter sequences ("CMV"),
30   bacterial lac Z sequences ("lac Z"), and SV40
polyadenylation signal ("pA") were inserted as a
cassette in place of the deleted E1A and E1B
sequences.
           Figure 2B is a map of pE1A. Adenovirus
35   type 5 nucleic acids are shown ("Ad 5") with
nucleotide numbers above and map units below the

indicated fragment. The Ad 5 nucleic acids were
ligated into the multiple cloning site of pUC 13
represented by hatched bars at the indicated
restriction endonuclease sites. "E" = *Eco* RI, "B" =
5   *Bst* 1107 I, "H" = *Hinc* II.

Figure 2C is a map of the AdCMVluc viral
genome. Black bar represents the deleted E1 region
sequences into which was inserted the luciferase
expression cassette consisting of the CMV promoter
10 ("CMV"), firefly luciferase coding sequence
("luc"), and an SV40 polyadenylation signal ("pA").

Figure 2D is a map of the adenoviral E1
region to indicate the viral sequences inserted
into the replication-enabling plasmids pE1A and
15 pE2206.

Figure 3 is a graphic representation of
the observed cytopathic effects produced by lysates
or supernatants from cells cotransduced with
AdCMVlacZ and plasmid DNA. Ordinate = qualitative
20 amount of cytopathic effect 48 hrs after addition
of the supernatants or lysates, abscissa = times of
lysate or supernatants after cotransduction. Key
indicates the plasmid cotransduced with the
adenovirus. The pUC 13 groups were observed up to
25 96 hrs after supernatant or lysate addition without
any cytopathic effect.

Figure 4 shows the luciferase transfer
capacity in the supernatants of HeLa cells
cotransduced with DOTAP/DNA complexes containing
30 AdCMVluc DNA and pE1A plasmid DNA. Shown are the
results 6 days after exposure to the DOTAP/DNA.

Figure 5 shows the luciferase activity
present in engrafted PC-3 tumors 10 days after they
were injected with either AdCMVluc and pE1A, or for
35 the controls, AdCMVluc and pUC 13.

Figures 6A and 6B are maps of the
retroviral nucleic acids.

Figure 6A is a map of pBAG. "LTR" = long
terminal repeat, "β-galactosidase" = bacterial lac
Z gene, "neo" = neomycin resistance gene, "pbr
origin" = plasmid origin of replication. Not to
5   scale.

Figure 6B is a schematic of retroviral
sequences within the pPAM3 plasmid. Open blocks at
left indicate the 5' LTR sequences, "SD" = splice
donor site, "SA" = splice acceptor site, "gag, pol,
10   env" = retroviral coding sequences, hatched bar =
SV40 polyadenylation signal. (Reproduced from
(Miller 1986 Molecular and Cellular Biology
6(8):2895.).

Figure 7 is a graphic representation of
15   the titers of retrovirus in supernatants of cells
cotransduced with pBAG and pPAM3. Supernatants
were collected 72 hrs post-transduction with the
complementary retroviral vectors. Ordinate = titer
as number of viral particles per ml using A549 as
20   indicator cells, abscissa = amounts of the 2
plasmids used to make the molecular conjugates.
The cell type cotransduced with the two plasmids
were A549 (circles) and PC-3 (triangles).

                 Detailed Description of the Invention
25   As used herein, "replication" is the
process of producing new viral particles that are
capable of infecting other cells. "Infection" is
the process of a viral particle binding to a
specific cell surface receptor resulting in the
30   release of the viral genome into the nucleus where
the viral genome directs the production of new
proteins.

As shown by Figure 1A, a replication-
competent virus infects a cell when the viral
35   particle binds to a receptor on the surface of the
cell and is transported into the cytoplasm and then

8

into the nucleus. Once in the nucleus, the
complete viral genome is transcribed, new viral
proteins are synthesized, and new viral particles
are made and released.

5   As shown by Figure 1B, in the case of a
replication incompetent virus, the viral particle
containing an incomplete genome also binds to a
cell surface receptor and is transported through
the cytoplasm into the nucleus. However, since the

10 viral genome is incapable of either being
transcribed, or directing synthesis of all viral
proteins required for packaging, infection with a
replication-defective virus does not lead to new
viral particle production.

15   The production of syngeneic, replication-
defective, recombinant virus-producing cells
requires the following steps. The fundamental
starting point is the requirement for a
replication-defective virus that can produce new

20 replication-defective virus in the presence of
additional genes provided in a trans configuration.
Although the specific designs of these different
viruses vary, as a general rule the recombinant
viral genome has some of the native viral genes

25 required for replication deleted, which may be
replaced by new genes of interest. Typically, the
replication-defective virus includes the 5' and 3'
LTRs and packaging signal sequences. This
replication-defective virus is generally referred

30 to herein as the "first nucleic acid sequence".
           The second step is the design of
complementing genomes that will be co-delivered to
the target cells in order to produce new
recombinant virus. These complementing genomes

35 must be present in the cells in which new
recombinant virus is to be produced but should
preferably not recombine with the defective virus

to produce replicating virus. The complementing
genome is generally referred to herein as the
"second nucleic acid sequence".

5   As part of either the first and/or second
steps, the biologically active genes of interest
which are to be delivered and expressed in the
target cells are incorporated into either, or both,
the first and second nucleic acid sequences, most
preferably the first nucleic acid sequence.

10   An optional third step is to provide
means for co-delivering the first and second
nucleic acid sequences.

            The final step is to co-deliver the first
and second nucleic acid sequences. This can be
15 accomplished by multiple, widely available gene
transduction methods. One then must establish that
co-delivery of the first and second nucleic acids
results in the production of new recombinant virus
which is infectious and capable of expressing the
20 incorporated genes of interest in the target cells.
The presence of new recombinant virus can be
assayed in the supernatant of the cells transduced
several days later, or within lysates of the
transduced cells. The identity of the new virus
25 can be established by the presence of marker genes,
or marker gene products, within the recombinant
virus genome.

Replication-Defective Virus ("RDV")

            Many replication-defective viruses,
30 particularly derived from retroviruses,
adenoviruses, adeno-associated viruses, and herpes
viruses, have been described in the literature and
are available from sources such as the American
Type Culture Collection, Rockville, Maryland.
35 These viruses are characterized as missing all or a
portion of one or more genes essential for
replication of the virus. In an appropriate cell

line, however, they are capable of forming
infectious virus particles. The viruses useful in
the methods described herein are those capable of
infecting and replicating in mammalian cells,
5 although they may infect certain cell types
preferentially.

Retroviruses are generally defined as a
family of eukaryotic viruses that replicate through
a DNA intermediate, as described by Panganiban 1985
10 Cell 42:5.

Herpes virus are classified based on a
characteristic virion architecture that includes an
icosadeltahedron forming the capsid with two-fold
symmetry surrounding the tegument that surrounds
15 the DNA genome, as described by Roizman in Human
Herpesviruses 1993 (Raven Press) pp. 1-9.

Adenovirus are nonenveloped viruses
containing a double stranded DNA genome with
characteristic antigenic properties and DNA
20 homology, as described by Wadell 1984 Current
Topics in Microbiology 110:191.

Parvoviridae are a family of DNA, non-
enveloped animal viruses containing a single
stranded DNA genome encapsulated within an
25 icosahedral protein coat composed of three proteins
with overlapping amino acid sequences. The family
includes three genera that includes (i)
parvoviruses (ii) adeno-associated viruses (AAV)
that usually require coinfection with adenovirus
30 (iii) densoviruses which multiply in insects, as
described by Berns 1990 Microbiological Reviews
54:316.

Recombinant retroviruses are generally
made by manipulating the proviral form of the
35 virus, i.e., the double stranded DNA copy of the
viral RNA genome. The starting virus is commonly
the Maloney murine leukemia virus that is available

11

from ATCC, Rockville, MD. Examples include VR-861,
VR-860, VR-590, and VR-589. The proviral DNA is
placed within a plasmid to permit amplification of
the DNA in bacteria. Once this has been
5   accomplished, the gag, pol and env genes are
excised by appropriate restriction endonucleases to
leave the 5' and 3' long terminal repeats (LTRs),
and the packaging signal sequences that are
immediately 3' to the 5' LTR. Multiple, detailed
10   descriptions of these constructions, as well as
permutations of this general scheme, are clearly
described in the scientific literature, for
example, by Cepko 1984 Cell 37:1053; Hwang 1984 J.
Virol. 50:417; Yu 1986 Proc. Natl. Acad. Sci. USA
15   83:3194; Armentano 1987 J. Virol. 61:1647; Yee 1987
Proc. Natl. Acad. Sci. USA 84:5197; Hawley 1989
Nuc. Acids Res. 17:4001; Miller 1989 BioTechniques
7:980.
        Recombinant adenoviruses are generally
20   made by manipulating the adenoviral DNA within
plasmids to permit amplification of the DNA in
bacteria. To date, the human serotypes 2 and 5
have been used for gene therapy purposes, largely
because their biology and genomes have been most
25   extensively characterized. The wild type
adenovirus type 2 and 5 are readily available from
the ATCC, Rockville, MD: Type 2, VR-846 and VR-
1079; Type 5, VR-5 and VR-1082. Most commonly, the
E1A region is deleted using convenient restriction
30   endonuclease sites within the E1A region. Often, a
portion of E3 is also deleted by restriction
endonuclease addition so as to permit the insertion
of a larger piece of foreign DNA while still
satisfying the size constraints required for
35   packaging into new viral particles. The details of
adenoviral vector constructions are widely
described in the literature, for example, by

Berkner 1984 <u>Nuc. Acids Res.</u> 11:6003; Ghosh-
Choudhury 1987 <u>Biochem. Biophys. Res. Commun.</u>
147:964; Gilardi 1990 <u>FEBS</u> 267:60; Mittal 1993
<u>Virus Res.</u> 28:67; Yang 1993 <u>Proc. Natl. Acad. Sci.</u>

5   <u>USA</u> 90:4601.

Traditionally, these replication-
defective viruses have been replicated through the
use of packaging cell lines. These cell lines
contain, at a minimum, the nucleic acid sequence

10   obtained from replicating virus that will
complement in trans the replication-defective viral
genome, resulting in new replication-defective
virus. A packaging cell line is made by stably
introducing the missing viral genes that are

15   required for replication. Recombinant virus is
made by introducing the recombinant viral genome
into the packaging cell line, the viral genome
(minus the genes present in trans that complement)
is replicated and packaged into viral particles

20   that can infect any cell type with the required
viral receptor. However, since the virus is still
replication-defective, the viral particles are
incapable of directing the production of new virus.

It has not previously been established

25   whether other elements in the specific packaging
cell lines might contribute to the production of
virus in unpredicted or unexpected ways. For
example, it is helpful to briefly consider the 293
cell line used for recombinant adenovirus

30   production. These cells were made by the
introduction of randomly sheared adenoviral DNA
that has been only partially characterized (Graham
1977 <u>Journal of General Virology</u> 36:59; Aiello 1979
<u>Virology</u> 94:460). Although it has been shown that

35   these cells contain E1A genes, and are capable of
"packaging" E1A-deleted recombinant adenoviruses,
it was never established whether other sequences

13

also present played a role, whether the copy number
of the sheared viral nucleic acid sequences present
in each cell was critical to the utility of this
specific cell line, or whether the specific parent

5   cell type contributed to the virus production.
Similarly, although the retroviral nucleic acids
used for establishing the PA317 packaging cell line
were precisely identified (Miller 1986), it was
unclear whether these sequences had to be stably

10   integrated within a cell line before the addition
of the recombinant viral genome, i.e., it was never
established that co-delivery of the two
complementing genomes would result in the
production of recombinant viral particles.

15   Some recombinant viral vectors are used
to produce recombinant virus particles by
coinfection with a helper virus, but the specific
helper virus genes that are required to
replication-enable the viral vector have not been

20   defined. Specific examples of such vectors include
the adeno-associated virus vectors described by
Nahreini 1993 Gene 124:257; and Samulski 1989 J.
Virol. 63:3822, and herpes vectors, described by
Geller 1988 Science 241:1667; and Breakfield 1991

25   New Biol. 3:203.

            A replication-defective adenoviral genome
was delivered by viable virus in the specific
examples described below. The same nucleic acids
alternatively could be delivered in a plasmid form,

30   since the methods for propagation of part (Berkner
1983 Nucleic Acids Research 11:6003), or all of the
adenoviral genome (Graham 1984 EMBO Journal 3:2917)
within plasmid DNA have been previously described.

14

**Genes required for replication of replication-
defective virus**

      In general, it is a straightforward
matter to supply the genes missing in the
5   replication-defective virus (RDV) in order to
provide a second nucleic acid sequence that
operates in trans with the RDV to replicate the
RDV. Although the second nucleic acid sequence can
include nucleic acid sequence also present in the
10   RDV, it is preferred that there not be overlapping
sequence since this can cause an undetermined
amount of recombination, leading to wild type virus
capable of replicating. This is not in itself
necessarily a problem, however. As referred to
15   herein, the second nucleic acid sequence includes
at a minimum the genes required for replication of
the replication-defective virus and means for
amplification thereof. It may optionally include
genes of interest, such as marker genes, suicide
20   genes, and therapeutic genes, as described in more
detail below. Although referred to as a
"sequence", the parts of the second nucleic acid
sequence can be present in one or more molecules,
usually plasmids, preferably a single plasmid.
25   In general, the genes required for
replication ("GRR") can be obtained by amplifying
some or all of the viral nucleic acids present in a
packaging cell line useful for replicating the RDV.
The GRR can also be obtained by excision from virus
30   that is capable of replication. For example, in
the case of recombinant adenovirus, the viral
nucleic acids that contain the E1A region are
excised, subcloned and amplified by routine
techniques. These nucleic acids complement the
35   deleted E1A sequences in common recombinant
adenoviruses heretofore only provided by packaging
cell lines, such as the 293 cell line, for the

purpose of replicating the viruses. In the case of
recombinant retrovirus, the nucleic acids that are
used to make the packaging cell line are obtained
in plasmid form and amplified with the intent of
5   co-delivery with the recombinant retroviral vector
plasmid.

The second nucleic acid sequence must be
provided in a form that can be amplified, although
it is preferably not in a form that infects cells
10 other than the targeted cells which are transduced
with the first nucleic acid sequences. By using a
second nucleic acid sequence which is limited to
defined cells, replication and infection of cells
can be controlled. This is particularly important
15 in the case where the genes of interest
incorporated with the first and/or second nucleic
acid sequences result in the death of the host
cells. The most preferred form therefore is a
plasmid. However, the second nucleic acid could
20 also be provided within a recombinant virus,
preferably one utilizing different receptors than a
virus containing the first nucleic acid.
Alternatively, the replication-enabling plasmid
could also be provided in the form of ribonucleic
25 acid. In all cases, the second nucleic acid must
include genes with the necessary transcription
activating and terminating elements necessary to
transcribe mRNA that can be translated into
proteins capable of enabling replication.
30   In the preferred embodiment where the
means for amplification of the GRR is a plasmid,
the plasmid promoter should allow replication in
the cells to be transduced with the second nucleic
acid sequence. The promoter may be cell type or
35 tissue type specific as another means for
controlling which cells are targeted.

The E1A genes required for replication of
adenovirus are provided in the pE1A plasmid as
described in the following examples. The
construction of this plasmid is described in detail
5   in the example using pEco RIA plasmid described by
Berkner 1984 Nuc. Acids Res. However, the E1A
fragment or other complementing fragments that
might be needed for replication-enabling other
adenoviral vectors, could easily be derived from
10   adenoviral DNA that is harvested from adenovirus-
infected 293 cells, or other adenoviral-permissive
host cells.

The genes required for replication of
retrovirus are provided in the pPAM3 plasmid
15   described in the following examples. The
construction of this latter plasmid is described in
detail by Miller 1986 Mol. Cell. Biol. 6:2895.
This description could be applied by one of
ordinary skill in the art to construct an
20   equivalent plasmid from the murine leukemia virus
proviral DNA.

**Genes to be incorporated into First or Second
Nucleic Acids**

Nucleic acids useful for gene therapy
25   include those that code for proteins used to
identify cells infected with the recombinant virus,
those that encode for proteins that function to
kill cells containing the viral genome, or that
encode for therapeutic proteins that will serve to
30   treat a pathophysiologic condition within the body.

The sequences that encode for many of
these proteins are known and published in the
literature. Representative marker genes include
those described in detail in the following
35   examples, including an enzyme such as ß
galactosidase and proteins conferring antibiotic
resistance or susceptibility. Other examples

17

include proteins that augment or suppress abnormal
proteins, as well as those that are toxic or
deleterious to abnormal cells within the body. One
example of the latter is the herpes simplex virus
5   thymidine kinase gene. The addition of ganciclovir
to cells expressing this gene results in death of
the cell. Still others are those which are
defective or missing in the patient to be treated,
for example, the cystic fibrosis transmembrane
10   regulator gene ("CFTR") can be added to cells
containing mutant CFTR with subsequent correction
of the ion transport defect caused by the mutant
CFTR gene. Examples of other genes currently being
investigated for use in gene therapy include
15   adenosine deaminase, insulin, coagulation factors
such as factor VIII, and glycogen degrading
enzymes.

          Although the sequences incorporated into
the first and/or second nucleic acids will
20   typically be nucleic acids encoding proteins, the
sequences themselves may also be biologically
active. Many examples of such materials are known,
for example, antisense and ribozymes. Unless
specifically stated otherwise, the genes encode
25   therapeutic molecules including biologically active
nucleic acids, nucleic acids encoding biologically
active proteins, and nucleic acids encoding
proteins responsible for producing the biologically
active molecules of interest, whether protein or
30   other type of molecule.

          For example, for human gene therapy use,
one of the retroviral nucleic acid constructs could
be modified to contain a "suicide gene" so that the
virus producing cells could be eliminated as
35   desired. As one specific example, the neomycin
resistance coding sequences could be excised from
pBAG, described in detail in the following

examples, with appropriate restriction endonucleases and replaced with herpes simplex virus thymidine kinase coding sequences. It is well established that cells expressing the viral

5   thymidine kinase gene product can be eliminated by treatment with the antiviral agent, ganciclovir (Moolten 1986 Cancer Research 46:5276). In this manner, the virus producing cells would be eliminated by systemic administration of FDA-

10   approved ganciclovir.

Means for Co-Delivery of First and Second Nucleic Acid Sequences

As described herein, the method for gene therapy can be *in vivo*, i.e., administered directly

15   to a patient for expression of a gene as defined above or for targeted killing of cells, or *in vitro*, for administration directly to cells outside of the body, for expression of exogenous genes. In a preferred example of the latter, cells are

20   obtained from a patient, the first and second nucleic acid sequences administered to the cells, and the cells returned to the patient. An example of this method is the treatment of stem cells or progenitor cells obtained from a patient following

25   cytokine administration to enhance proliferation and mobilization of the stem cells and progenitor cells in the peripheral blood. The genetically engineered cells are then returned to the patient. Alternatively, the cells can be maintained in

30   culture for production of the molecules encoded by the exogenous genes carried by either the first and/or second nucleic acid sequences.

In general, preferred means for co-delivery include the use of (1) a first nucleic

35   acid sequence including infective replication-defective virus in combination with the second nucleic acid sequence, (2) the first nucleic acid

sequence ionically or covalently coupled to the
second nucleic acid sequence, alone or in further
combination with an enhancer of transduction, (3)
intact infective virus in combination with the
5   first nucleic acid sequences coupled with the
second nucleic acid, where the intact virus is
rendered non-viable after infection by ultraviolet
irradiation in the presence of 8-methoxypsoralen or
other free radical initiators such as methylene
10   blue, and (4) delivery of both the first and second
nucleic acid sequences separately, by viruses
utilizing different receptors.

     In the preferred embodiment, the first
and second nucleic acid sequences are introduced
15   simultaneously into the cells where virus is to be
replicated. The first nucleic acid within a virus
is delivered to the cells by the viral infection
medium, and the second nucleic acid is added
separately by a non-viral means, for example, naked
20   DNA administration or coupled with an enhancer of
transduction. The second nucleic acid can also be
coupled to the virus containing the first nucleic
acid by means of an agent on the viral surface
having an affinity for nucleic acids, such as
25   polylysine, followed by the addition of free agent
to further condense the second nucleic acid, after
which the entire complex is administered to the
cells. Alternatively, the second nucleic acid can
be coupled to an inactivated virus by means of an
30   agent on the viral surface having an affinity for
nucleic acids by first inactivating the virus
followed by all the other steps used to make a
complex as described above. The first and second
nucleic acids can also be coadministered by
35   viruses utilizing different receptor types, a
requirement to avoid receptor-interference.

20

<u>Infection with the defective retrovirus:</u>
Retroviral-mediated delivery of the
sequences required for replication of defective
viruses can be accomplished using retroviral
5   vectors or other vectors known to those skilled in
the art. For example, E1A sequences can be
delivered using the pLN retroviral vectors
developed by Miller and colleagues (Miller 1989
<u>BioTechniques</u> 7:980), provided A.B. Miller, Fred
10   Hutchinson Cancer Research Center, Seattle, WA.
The desired E1A sequences are excised with
appropriate restriction endonucleases from a parent
plasmid construct, blunted and ligated into a
similarly blunted cloning site of pLNSX, described
15   by Miller, 1989 <u>BioTechniques</u> 7:980. Since pLNSX
contains an SV40 early promoter upstream of the
cloning site, this can be excised by a *Bam* HI/*Hind*
III digest at the time of linearizing the vector,
after which the sites are be blunted. The method
20   described by Butterworth and Miller (Miller 1986)
can be used in which the plasmid retroviral vector
containing the E1A sequences is transfected into
the Psi2 ecotropic packaging line described by
Cepko 1984 <u>Cell</u> 37:1053, without selection. The
25   transient supernatant containing some ecotropic
virus is collected 48 hours later and used to
infect the PA317 ecotropic packaging cell line by
Miller 1986 <u>Mol. Cell Biol.</u> 6:2895. Individual
producer clones are selected by culturing the cells
30   in G418 media, amplifying and testing for titer by
methods described by Rousculp 1992 <u>Human Gene
Therapy</u> 5:471.

<u>Co-delivery of first and second nucleic
acid sequences:</u>
35   Examples of materials facilitating co-
delivery of the first and second nucleic acid
sequences include charged molecules that ionically
couple the nucleic acid sequences, such as

polylysine, as well as biotin conjugation with
streptavidin and other cross-linking agents that
can be used with standard technology to covalently
couple the first and second nucleic acid sequences.

5   Coupling by means of an agent ionically
binding nucleic acid, such as polylysine, is
described by the U.S. patent application entitled
"Composition for Introducing Nucleic Acid Complexes
into Higher Eucaryotic Cells" filed September 2,

10   1992 by David T. Curiel, Max L. Birnstiel, Matthew
Cotten, Ernst Wagner, Kurt Zatloukal, Christian
Plank, and Bernd Oberhauser, the teachings of which
are incorporated herein.

          DNA can be coupled using a covalent

15   crosslinking agent, for example, polyglutaraldehyde
(Digene Diagnostics), or more typically, by
biotinylation and linkage via an avidin
(streptavidin) bridge to the viral surface.
Biotinylation is well known to those skilled in the

20   art, for example, as described by Avignolo, et al.,
1990 <u>Biochem. Biophys. Res. Commun.</u> 170:243-250.

          Example 1 demonstrates a preferred
embodiment for co-delivery of pE1A (a second
nucleic acid sequence consisting of a plasmid and

25   the GRR for a replication-defective adenovirus) and
AdCMVlacZ (a replication-defective adenovirus
including the CMV promoter and lacZ bacterial gene
encoding ß galactosidase as a marker gene)
accomplished by ionically linking the pE1A to the

30   virus exterior. Figure 2A is a map of the
AdCMVlacZ viral genome. Hatched bars represent the
conserved human type 5 adenovirus sequences with
map units indicated below. CMV promoter sequences
("CMV"), bacterial lac Z sequences ("lac Z"), and

35   SV40 polyadenylation signal ("pA") were inserted as
a cassette in place of the deleted E1A and E1B
sequences. Figure 2B is a map of pE1A. Adenovirus

22

type 5 nucleic acids are shown ("Ad 5") with
nucleotide numbers above and map units below the
indicated fragment. The Ad 5 nucleic acids were
ligated into the multiple cloning site of pUC 13
5   represented by hatched bars at the indicated
restriction endonuclease sites.

        Molecular conjugates can also be used to
deliver the E1A nucleic acid sequences. These
consist of a means for ionically linking the first
10   and second nucleic acid sequence via a covalently
coupled conjugate. One example is the polylysine-
conjugated transferrin (TfpL) commercially
available from SIGMA Chemical Co., St. Louis, MO
(Cat. # T 0288). The optimal proportions of
15   nucleic acid to TfpL are established in preliminary
experiments with a reporter plasmid. The E1A
sequences are mixed with the TfpL and added to the
target cells.

                Infection with a virus which is then
20   rendered non-viable

        A virus capable of normal infection can
be rendered non-viable by exposure to a dye such as
8-methoxypsoralen which kills virus in the presence
of ultraviolet radiation, as described in detail by
25   Cotten, et al., 1992 Proc. Natl. Acad. Sci. (USA)
89:6094, after which the first and/or second
nucleic acids can be coupled to the inactivated
surface as described above.

        Other useful materials include methylene
30   blue and other free radical initiators and dyes
that are known to selectively damage viral DNA.

                Transduction Enhancers

        The first and second nucleic acid
sequences may be transduced separately or together
35   into the cells where viral replication is to occur.
There are many methods for transduction of cells
with viral material that are known to those skilled
in the art, for example, Gene transfer and

expression: a laboratory manual Kriegler M. 242 pp.
(W.H. Freeman, NY 1991); and Current Protocols in
Molecular Biology, 1987-1994, Ausubel F.M., et al.,
section entitled "Introduction of DNA into
5   Mammalian Cells" pp. 9.0.1-9.17.2 (John Wiley &
Sons).

        In a preferred example of a transduction
enhancer, cationic lipids, which typically are
phospholipids modified to obtain a positive charge,
10   are used for transduction of the first and second
nucleic acid sequences. A number of lipid
compounds shown to have efficacy for nucleic acid
transduction, for example, the cationic lipid N-[1-
(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-
15   ammoniummethylsulfate (DOTAP), can be used. The
stock solution commercially obtained from
Boehringer Mannheim is diluted three-fold with
HEPES buffered saline and mixed with DNA at 50
ng/μl. The typical ratio of lipid to DNA
20   (weight:weight) is 6:1, but other ratios may
provide better results, as determined by empiric
testing. After allowing the DNA and lipid to
associate for 10 minutes, the lipid-DNA complexes
are administered to the target cells.
25   In each of these alternative delivery
methods, the precise conditions for the
cotransduction can be easily established by those
skilled in the art using the teachings provided
here. In certain cell types, optimal results are
30   obtained by separating the infection with the virus
from the transduction of the complementing GRR
sequences. In addition, some cell types or
physiologic situations may produce optimal results
with a specific means of GRR nucleic acid delivery.
35   Furthermore, it is possible that multiple
transductions of the GRR and/or the RDV may result
in additional virus production.

24

Examples

Co-transduction of replication-defective
adenovirus

Co-transduction of replication-defective
5   adenovirus with the required nucleic acid sequences
for replication was employed in the following
examples, demonstrating the successful development
of compositions and methods for making recombinant
adenovirus from cotransduced cells that prior to
10   transduction did not make any virus or contain any
virus nucleic acids. The recombinant adenovirus
employed was AdCMVlacZ (Yang 1993 Proc. Natl. Acad.
Sci. (USA) 90:4601), an E1A-deleted virus (map
units 1.3 - 9.4 deleted) that included a CMV
15   promoter-driven, bacterial lac Z gene. A viral
stock of AdCMVlacZ was amplified in 293 cells and
modified to contain polylysine molecules on the
virus exterior using methods described in detail in
Example 1. A plasmid, designated pE1A, was
20   designed to contain the E1A nucleic acids deleted
from AdCMVlacZ. The pE1A plasmid contained
nucleotides 1- 5768 of the human adenovirus type 5
genome that included E1A and E1B. Although the
pE1A plasmid was derived from another adenoviral
25   plasmid, the required nucleic acids could be easily
derived from adenoviral infected cells, or directly
from adenoviral DNA that does not contain any
deletions in the E1A region.

The possibility of recombination can be
30   eliminated, or greatly reduced, by making a
complementary plasmid that contains the bare
minimum of sequences necessary to complement those
missing from AdCMVlacZ, or related E1A-deleted
viruses. Although the E1A region encodes proteins
35   of 243 and 289 amino acids (Flint 1989 Ann. Rev.
Genetics 23:141), previous work has shown that the
289 amino acid protein alone is sufficient for
trans activating the other viral genes and

25

establishing lytic infection (Winberg 1984 EMBO
Journal 3:1907; Moran 1986 J. Virology 57:765).
Very recently, exon 2 of the E1A region was shown
to be sufficient for transactivation of the other
5   viral genes, although the degree of activation was
variable in different cell lines (Mymryk 1993 J.
Virology 67:6922). Therefore, one can utilize
smaller portions of the E1A region than employed in
the following examples. The variation of trans
10   activation by exon 2 of E1A in different cell types
can also be used to target viral replication to
cell types in which exon 2 of E1A alone is a
sufficient trans activator.

Although one recombinant adenovirus is
15   used in the following examples, it is evident that
the approach can be used for trans complementation
of other adenoviral genes, and other adenoviral
serotypes. For example, fibre-deleted adenoviral
mutants and methods for propagating those mutants
20   have been described by Falgout 1987 J. Virology
61:3759; Falgout 1988 J. Virology 62:622. In the
case of the fibre-deleted mutant, the strategy
would employ the co-delivery of the recombinant
virus with the deleted fibre gene and a separate
25   nucleic acid encoding the deleted fibre sequences.
Since trans complementation of replication-
defective adenoviruses using cell lines that
contain complementing nucleic acids has also been
described for E4 and E2A deletion mutants (Weinberg
30   1983 Proc. Natl. Acad. Sci. (USA) 80:5383; Klessig
1984 Mol. Cell. Biol. 4:1354), these are additional
replication-defective adenoviruses that could be
complemented in trans using the methods described
here. Furthermore, this type of approach could be
35   used to mix genes of different adenoviral serotypes
to produce new tropisms or other biological
effects.

26

Co-transduction of replication-defective retrovirus

Recombinant retrovirus was also produced by cells cotransduced with recombinant viral and
5 complementing viral nucleic acid sequences. The recombinant retroviral vector used in the example shown here, pBAG, contains a bacterial lac Z gene and a neomycin resistance gene in place of deleted viral genes gag, pol and env (Price 1987 Proc.
10 Natl. Acad. Sci. (USA) 84:156). A plasmid vector containing the deleted retroviral genes, pPAM3, was chosen as the complementing nucleic acid because this plasmid had been used to create the widely used packaging cell line, PA317 (Miller 1986).
15 Both the vector and complementing plasmids were co-delivered by ionic linkage to polylysine conjugated to the exterior of the DL1014 adenovirus (Bridge 1989 J. Virology 63:631) using the methods detailed in Example 2.

20 ## Co-transduction of retrovirus and adenovirus genes

The examples described below are specific to either replication-defective adenovirus or to replication-defective retrovirus. An advantage of
25 retrovirus is that it only infects replicating cells, as reported by Miller 1990 Molecular and Cellular Biology 10(8):4239. This feature has been used to target replicating cells in a therapeutic context, as described by Culver 1992 Science
30 256:1550. This is particularly useful in cancer treatment since the tumor cells typically replicate more rapidly than the normal host cells. In the brain, the tumor cells are typically the only replicating cells.

35 Accordingly, one can use a combination of retrovirus and adenovirus to replicate replication-defective virus selectively in replicating cells. The retrovirus still infect only replicating cells.

27

It is used as a carrier for the GRR for adenovirus
(E1A). The cells are also infected with
replication-defective adenovirus. Since the
purpose of infecting the cells is to kill the
5   cells, and infection and replication of adenovirus
in cells results in cell lysis and death, no
additional non-viral genes are required to kill the
targeted cells.

In addition to the methods described
10 above, recombinant adenoviruses containing one or
both of the complementary retroviral nucleic acid
sequences can also be constructed and used. In
this case, the replication-defective virus is a
retrovirus and the replication-defective adenovirus
15 is a carrier for the GRR for the retrovirus. The
methods described by Graham and Prevec (1991)
Methods in Molecular Biology. Clifton, The Humana
Press Inc. 109, are used to create replication-
defective adenoviruses containing the retroviral
20 nucleic acids at the site of the E1A deletion. In
brief, the retroviral nucleic acid sequences are
ligated into a blunted cloning site of pXCJL-2
plasmid that contains the E1A deletion portion of
the virus. Standard techniques are used for the
25 plasmid construction and identification. The
recombinant virus is made by co-transfecting the
293 cell line (which provides E1A viral proteins in
trans) with the pJM17 vector that is designed to
homologously recombine with the pXCJL plasmid
30 derivatives to produce a full length, packagable
viral transcript lacking E1A, as described by
Graham 1991.

The following non-limiting examples
demonstrate actual reduction to practice of the
35 methods and compositions described herein, as well
as preferred embodiments.

28

Example 1.   **Creation of Syngeneic Recombinant**
                        Virus-Producing Cells by
                        Cotransduction of a Replication -
                        Defective Virus and a Replication-
5   **Enabling Plasmid**

          a.   <u>Construction of the Replication-</u>
                 <u>Enabling Plasmid</u>

          A plasmid was constructed to contain the
region of adenoviral genome deleted in a
10   replication-defective adenovirus. The replication-
defective, human adenovirus, AdCMVlacZ, was unable
to replicate in most cells because the lac Z gene
had been inserted within the deleted E1A region of
the viral genome, as described by Yang 1993. A
15   second replication-defective human adenovirus also
used was AdCMVluc which contains a firefly
luciferase gene within the deleted E1A region (Herz
1993 <u>Proc. Natl. Acad. Sci. USA</u> 90:2812). The maps
of AdCMVlacz and AdCMVluc are shown in Figure 2.
20   Therefore, the E1A adenoviral region was isolated
and inserted into a plasmid vector.

          The E1A region was isolated from the
plasmid pEcoRIA containing nucleotides 1-27331 of
human type 5 adenovirus (Berkner 1983 <u>Nucleic Acids</u>
25   <u>Research</u> 11:6003). Although the pEcoRIA plasmid
contained the desired E1A region, it was found in
the course of experimentation to undergo frequent
recombination events in the course of routine
plasmid amplification that reduced its usefulness
30   for the desired application. Adenoviral nucleotide
sequences 1-5768 were excised from pEcoRIA by
restriction endonucleases <i>Eco</i> RI and <i>Bst</i> 1107 I.
The fragment was isolated from the parent nucleic
acid by agarose gel electrophoresis and extracted
35   from the gel using the GeneClean™ II Kit (Bio 101,
La Jolla, CA). The blunted E1A-containing fragment
was ligated to the <i>Eco</i> RI/<i>Hinc</i> II site of pUC 13
within the multiple cloning site of that vector.
Identity of the final plasmid, designated pE1A (map

shown in Figure 2), was confirmed by restriction
endonuclease analysis that compared observed
fragment sizes to those predicted by a computer
generated restriction endonuclease map of that
5   fragment.

A second replication-enabling plasmid was
made to contain a smaller portion of the adenoviral
E1A region. The *Eco* RI and *Bst* 1107 I fragment
containing adenoviral nucleotide sequences 1-5768
10 was cleaved with *Nco* I to release a fragment
containing adenoviral nucleotide sequences 1-2206.
This fragment was blunted and ligated to the
blunted *Eco* RI/*Hinc* II site of pUC 13 within the
multiple cloning site of that vector. Identity of
15 the plasmid, designated pE2206 (map shown in Figure
2), was confirmed by restriction endonuclease
analysis.

b.   Production of Adenovirus to be Co-
      Delivered with Replication-Enabling
20   Plasmid

The AdCMVlacZ virus or AdCMVluc virus was
amplified by standard techniques by passage through
293 cells. The 293 cells were propagated by
routine tissue culture techniques to form an almost
25 confluent monolayer in 10-20, 175 cm² flasks. The
cells were inoculated with a stock of the AdCMVlacZ
at a multiplicity of infection of approximately 100
to 1000:1 for 2 hrs in a minimal volume of 2% fetal
calf serum-containing media. After this period,
30 the tissue culture flasks were supplemented with
10% fetal calf serum-containing media ("regular
media") at twice the inoculating volume. Three
days later, the infected cells were scraped off the
plate and isolated by centrifugation (8000 RPM at
35 4°C for 30 min in a Beckman JA-17™ rotor). The
cell pellet was resuspended in between 5 and 10 ml
of regular media, after which the cells were lysed
by four consecutive freeze-thaw cycles. The lysate

30

was clarified by centrifugation (8000 RPM at 4° for
30 min in Beckman JA-17™ rotor) and layered on top
of a cesium chloride gradient in Beckman SW-28™
centrifuge tubes. The gradient consisted of 20 ml
5   1.33 gm/ml cesium chloride in 5 mM HEPES on top of
a 10 ml 1.45 gm/ml cesium chloride in 5 mM HEPES
cushion. Typically, between 6 and 7 ml of lysate
was applied to each tube. The viral particles were
concentrated within the gradient by spinning the
10 tubes for 90 min at 20°C at 18,000 RPM. The viral
band was extracted by a syringe needle introduced
into the side of the tube. The aspirated virus was
diluted with an equal volume of 5 mM HEPES, pH 7.8
and applied to the top of a cesium chloride
15 gradient within Beckman SW 41™ tubes. The gradient
in these tubes consisted of 3.5 ml 1.33 gm/ml
cesium chloride in 5 mM HEPES on top of a 3.5 ml
1.45 gm/ml cesium chloride in 5 mM HEPES cushion.
Typically, each tube received between 4 and 4.5 ml
20 of the virus isolated from the first gradient spin.
The viral particles were concentrated within the
gradient by spinning the tubes for 18 hrs at 20°C
at 26,000 RPM. After this spin, there was usually
a prominent lower band and a fainter upper band.
25 The lower band was aspirated with a syringe needle
as described after the first gradient spin.

As one approach to co-delivering the
adenovirus and the pE1A plasmid, the adenovirus was
prepared to contain a moiety capable of binding
30 plasmid DNA to its exterior. To this end,
polylysine was attached to the exterior of the
adenovirus isolated as described below. The
aspirated virus was adjusted to a volume of 2.5 ml
by the addition of 1.33 gm/ml cesium chloride in 5
35 Mm HEPES. A PD-10™ column (Pharmacia, Piscataway,
NJ) was equilibrated with HBS (20 Mm HEPES Ph 7.8,
150 Mm NaCl). The viral solution was loaded onto

31

the PD-10 column by gravity, then eluted with 2 ml
of HBS. The column eluate was adjusted to a volume
of 3.6 ml by adding HBS. Polylysine solution was
made by dissolving 100 mg of polylysine (SIGMA #P-
5 2636) in 10 ml HBS, adjusting Ph to 7.8 with NaOH,
followed by adjusting total volume to 16.8 ml by
the addition of HBS. 0.4 ml of the final
polylysine solution was added to the 3.6 ml of
viral eluate. EDC linker solution was made by
10 dissolving 1 gm EDC (Pierce, #22980G) in a total
volume of 4 ml distilled water. 40 μl of the final
EDC solution was added to the polylysine/virus
mixture, rapidly but gently mixed with a pipet,
then the mixture incubated on ice for 4 hrs.
15 Following this incubation, 8 ml of cesium chloride
in 5 mM HEPES was added, mixed by pipet, and the
virus concentrated by centrifugation in an SW 41™
rotor at 25,000 RPM for 18 hrs at 20°C. The viral
band is again aspirated by a syringe needle as
20 previously described and diluted with an equal
volume of viral preservation media (50 % glycerol,
10 mM Tris pH 8.0, 100 mM NaCl, 1 mg bovine serum
albumin/ml). The number of viral particles was
estimated by spectroscopy at 260 mM in which 1 O.D.
25 = 1 X 10^{12} viral particles. The virus was stored in
aliquots at -70°C until further use.

    c.   Testing of the Polylysine-Conjugated
          Adenovirus

        It was necessary to test the conjugated
30 adenovirus to establish the optimal number of viral
particles and amount of plasmid to effect maximal
expression of coding sequences within the plasmid.
To do this, a range of viral particle numbers:
0.75 X 10^9, 1 X 10^{10}, and 2.5 X 10^{10} were diluted to
35 a total volume of 250 μl by the addition of viral
preservation medium. Six micrograms of the
constitutively expressed reporter plasmid, pGL2
control (Promega, Madison, WI) was added to a total

volume of 250 μl HBS, and this mixture added to the viral particles. The DNA was allowed to complex with the polylysine conjugated to the adenovirus for 30 min at room temperature, after which 4 μg of free polylysine (stock solution = 1 μg/μl in distilled water) was added to 246 μl of HBS, and the mixture added to the DNA/adenovirus mix. The free polylysine was permitted to further complex with the DNA/adenovirus complex so as to further condense the DNA for an additional 30 min at room temperature. Groups received either 9 or 12 μg of plasmid DNA and either 6 or 8 μg of free polylysine in order to examine whether higher amounts of plasmid per viral particle resulted in better expression of the reporter gene within the plasmid.

The final complexes of adenovirus and DNA were applied to cells that were subsequently tested for reporter gene expression. The cell lines HeLa, A549, or others were typically plated at densities of 1.5-2.0 X 10^5/35 mm culture dish the night before the complexes were added. Each plate received 1/10 of the total complex (i.e., 75 μl) in 1 ml of 2% fetal calf serum. Following a 2 hr incubation under usual culture conditions of temperature, humidity and CO_2, the plates were washed 3 times with phosphate buffered saline and replaced with regular media. Two days later, cell lysates were harvested and analyzed for luciferase activity using the instructions and reagents of the Luciferase Assay System, Promega. It was commonly observed that 2.5 X 10^{10} viral particles with 6 μg of plasmid DNA produced the optimal expression of the reporter gene.

33

d.   Demonstration of New Adenovirus by
      Cells Infected with Adenovirus and
      Simultaneously Transduced with the
      Replication-Enabling Plasmid

Experimentation established that cells
infected with an E1A-defective virus and
simultaneously transduced with an E1A-containing
plasmid in trans resulted in the production of new
virus by the infected cells. The pE1A plasmid was
complexed to the exterior of the polylysine-
conjugated AdCMVlacZ using the optimized viral
particle numbers, plasmid amounts, and methods as
described above. As a control in these
experiments, the polylysine-conjugated AdCMVlacZ
was complexed with the pUC 13 plasmid that did not
contain any adenoviral sequences. The complexes
were applied to the human prostate adenocarcinoma
cell line, PC-3, that had been plated at a density
of $1.5-2.0 \times 10^5$ cells/35 mm plate. Each plate
received 1/10 of a complex in quadruplicate under
conditions described in the previous section. Two
hrs later, the plates were washed thrice with
phosphate buffered saline and regular medium
applied. Two, three and four days later,
supernatants and cell lysates were collected. The
supernatants were prepared by aspirating the media
from the plates for each group, clarifying by
centrifugation (1500 RPM at 4°c for 5 min) and 0.45
μM filtration, after which the media was stored at
-70°C. The lysates were prepared by scraping the
plates, then pooling the cells from each group in a
total volume of between 0.5 and 0.7 ml regular
media. The cells were exposed to four freeze-thaw
cycles, the supernatant clarified by centrifugation
(3000 RPM in microfuge for 5 min. at 4°C) and
frozen at -70°C.

The supernatants and lysates were tested
for the presence of new adenovirus by exposure to

34

293 cells.. If new replication-defective virus was
present, the 293 cells should develop the classic
cytopathic effect (CPE) of rounding and detachment
from the culture dish surface. Figure 3 is a
5   graphic representation of the observed cytopathic
effects produced by lysates or supernatants from
cells cotransduced with AdCMVlacZ and plasmid DNA.
The pUC 13 groups were observed up to 96 hrs after
supernatant or lysate addition without any
10   cytopathic effect. The lysates and supernatants
from the cells that had been exposed to the
AdCMVlacZ + pE1A all produced CPE in the 293 cells,
although the onset of CPE was faster with the
lysates than the supernatants at all harvest times.
15   In contrast, none of the lysates or supernatants
from the cells exposed to the AdCMVlacZ + pUC 13
developed any signs of CPE.

        Similar experiments using the human
ovarian carcinoma cell line, SK-OV-3, obtained from
20   ATCC, also yielded only supernatants and lysates
from the pE1A groups that produced CPE in 293
cells.

        Experimentation with the AdCMVluc virus
shows that new replication-defective virus was by
25   codelivery of virus and a replication-enabling
plasmid (Goldsmith 1994 Human Gene Ther. 5: 1341).
Table 1 shows the viral titers measured in the
supernatants of cells cotransduced with the
AdCMVluc virus and pE1A or pUC13 (control plasmid
30   not containing any replication-enabling sequences).
The pE1A plasmid, or the pE2206 plasmid, was
complexed to the exterior of the polylysine-
conjugated AdCMVluc using the optimized viral paral
particle numbers, plasmid amounts and methods as
35   described above. The same controls and
experimental conditions were used to apply the
viral/plasmid complexes to the PC-3 cells.

TABLE 1: TITERS IN COTRANSDUCED CELL SUPERNATANTS

Harvest time[b]	A[a]		B[1]		C[1]	
	pUC	pE1A	pUC	pE1A	pUC	pE1A
72	1.5×10^2	1.3×10^6	-	1.0×10^6	2.5×10^2	2.3×10^6
96	10.0×10^2	1.7×10^6	-	4.0×10^6	7.0×10^2	4.2×10^6
120	21.5×10^2	2.5×10^6	-	8.3×10^6	9.5×10^2	2.6×10^6

a   Letters denote separate experiments.

b   Hours after cotransduction that supernatants were harvested.

36

Adenoviral particles present in the
supernatants of the PC-3 cells cotransduced with
adenovirus and pE1A were quantified by titer assays
utilizing 293 cells. 293 cells were plated in 35
5   mm plates to produce 50-60% confluence the next
day. Duplicate dilutions of supernatant from the
cotransduced PC-3 cells were added to the 293 cells
for 4 hrs, followed by aspiration and a wash with
PBS, after which complete media was added. The
10   presence of wild type virus was assessed by
applying undiluted 10^{-1}, and 10^{-2} dilutions to HeLa
cells under identical conditions to those used for
the 293 cell titers. Twenty-four hours later, the
media was replaced by molten agar overlay (DMEM/F12
15   supplemented with 0.65% noble agar, 2% FCS, 25 mM
$MgCl_2$ antibiotics). The titer plates were fed by
the addition of equivalent amounts of agar overlay
every 3 days. Plaques were manually counted
independently by two observers seven days later.
20   The supernatants from cells cotransduced with the
E1A plasmid and AdCMV-luc had concentration
dependent numbers of plaques quantified by limiting
dilution to derive the figures of 1 to 8 x 10^2.
These results showed that the replication-enabling
25   plasmid caused at least a 4 log increase in new
recombinant virus production compared to the
controls that received only the pUC 13 plasmid.
Small amounts of presumed wild type
recombinant virus has been detected in a minority
30   of the experiments. In one of eight experiments,
plaques were detected in HeLa cells exposed to
supernatants from pE1A-cotransduced cells (72 hr:
1.1 x 10^1, 96 hr: 7.9 x 10^1, 120 hr: 1.5 x 10^1) and
no plaques from the pUC-cotransduced supernatants.
35   One other experiment looking only at the 72 hr time
point found only 4 plaques per ml. However, use of
the pE2206 replication-enabling plasmid has not

37

resulted in any wild type plaque formation in the
HeLa cells in seven out of seven experiments.

   The new virus made by the cells cotransduced
with the pE1A and AdCMVluc virus was shown to be
5   functionally similar to the starting virus based on
luciferase transfer assays (Goldsmith 1994 Human
Gene Ther. 5:1341). Table 2 shows the luciferase
transfer capacity of supernatants from PC-3 cells
cotransduced with the AdCMVluc virus and pE1A or
10   pUC13. Since the AdCMVluc virus contains the
capacity to transfer the functional luciferase gene
to new cells with subsequent luciferase production,
it would be expected that the if the new virus
present in the supernatant of the cotransduced
15   cells is AdCMVluc, it should also transfer
luciferase activity to new cells. Supernatants
were aspirated from cotransduced cells at the times
indicated in the text, clarified by centrifugation
and stored at -70°C until testing. Lysates from
20   the cotransduced cells were made by pooling all the
enzymatically detached cells from each group (4
plates) in 1.2 ml complete media, freeze-thawing
four times, clarifying the lysate by centrifugation
and storing at -70°C. Fresh PC-3 cells were plated
25   (1 X 10^5 cells/well in 24 well plates) the evening
prior to lysate or supernatant exposure. The cells
were exposed to 50 μl of lysate or 500 μl
supernatant for four hours in 0.5 ml low serum
media, after which complete media was added. The
30   cells were grown under the conditions described
above for 72 hours, and luciferase activity
measured in the lysates using reagents and
instructions of a kit (Luciferase Assay System,
Promega, Madison, WI). Protein concentration was
35   measured in the lysate from each individual well in
duplicate by the Bradford method using the reagents

and instructions of a kit (Biorad Laboratories, Melville, NY).

TABLE 2: Luciferase Activity in Cells Treated with Lysates or Supernatants from PC-3 Cotransduced with AdCMV-luc and Indicated Plasmids

Lysates

Harvest[b] time	A[a]		B[a]		Ca	
	pUC	pE1A	pUC	pE1A	pUC	pE1A
72	19[c](2.5)[d]	8,000(254)	20(0.9)	5,342(291)	6(0.9)	3,501(167)
96	7(0.6)	3,104(327)	10(3.8)	4,789(1,615)	8(3.0)	6,910(4,191)
120	9(0.5)	4,534(385)	11(1.4)	3,984(868)	5(1.0)	2,323(398)

Supernatants

Harvest[b] time	A		B		C	
	pUC	pE1A	pUC	pE1A	pUC	ppE1A
72	12(8)	11,438(1525)	15(8.5)	1,297(120)	210(103)	6,039(335)
96	176(50)	7,472(376)	11(1.2)	7,091(852)	91(42)	8,582(1,118)
120	163(49)	9,245(565)	85(22)	4,594(389)	6(3)	2,676(700)

a   Letters denote separate experiments.
b   Hours after cotransduction that lysate or supernatant were harvested.
c   All unbracketed numbers indicate mean RLU's/µg protein.
d   All bracketed numbers indicate ± SEM.

PC-3 cells that had been exposed to
lysates and supernatants of the pE1A cotransduced
cells had significant levels of luciferase activity
detected 72 hours later. The supernatants from all
5 three experiments consistently had greater
luciferase-transduction capacity than the lysate
from those cells. PC-3 cells exposed to either the
lysates or the supernatants of the pUC cotransduced
cells had barely detectable levels of luciferase
10 activity in all three experiments. Similar
analyses have also *been performed with human cell
lines SK-Lu1, SK-OV3, A549 and HeLa that had
markedly increased amounts of luciferase transfer
activity in groups cotransduced with AdCMVluc and
15 pE1A or pE2206 compared to the controls
cotransduced with virus and pUC13. Table 3 shows
the luciferase transfer capacity in the
supernatants of multiple cell lines cotransduced
with the AdCMVluc virus and pE1A pr pUC 13. These
20 additional experiments demonstrated that the
replication-enablement could work in multiple cell
types, not simply the PC-3 cells.

TABLE 3: Evidence of new Ad CMV luc virus
production by assessment of luciferase
transfer capacity in supernatants of
multiple cell lines exposed to Ad CMV luc
and pE2206.

Cell Line	Control[1] RLU's	pE2206 RLU's
HeLa	24.3	934.3
SKlu1	49.7	1301.3
A549	17.7	1310.3
SKOV3	10.3	289.0

As further evidence that new recombinant virus
had been made, adenovirus was recovered from the
AdCMVlacZ + pE1A-treated cells and shown to be
identical to the starting virus by DNA analysis.
5 Supernatants were collected, clarified by
centrifugation, and pooled from the 293 cells that
developed CPE following lysate or supernatant

41

exposure (from the virus + pE1A-treated groups).
The supernatant pool was applied to confluent
plates of fresh 293 cells; each 10 cm diameter dish
received 0.5 ml of supernatant in 4 ml of 2% fetal
5   calf serum media. The remainder of the adenovirus
amplification was performed as described above.
Following the second cesium chloride gradient, the
viral band was aspirated and exhaustively dialyzed
against 10 mM Tris pH 8.0 at 4°C. The virus was
10   added to an equal volume of 20 mM Tris pH 7.8, 10
mM EDTA pH 8.0, 1.0% SDS containing proteinase K at
100 μg/ml, then incubated at 37°C for between 2 and
16 hrs. The mixture was extracted once with an
equal volume of buffered phenol, and DNA
15   precipitated from the remaining solution with
ethanol and sodium acetate. The same steps were
repeated using the AdCMVlacZ viral stock in order
to provide the parent adenoviral DNA specimen for
comparison. The DNA pellets were resuspended in 10
20   mM Tris pH 8.0, 1 mM EDTA and concentration
determined by UV spectroscopy (A_{260}/A_{280}). Aliquots
(2 μg) of each specimen were cut with restriction
endonucleases Xho I and Sal I, the fragments size
fractionated on 1% agarose and the resulting bands
25   compared for relative size. An ethidium bromide-
stained photograph of the gel showed identical
fragments were produced by the two viral DNA
specimens: adenoviral DNA obtained from PC-3 cells
following cotransduction with AdCMVlacZ and pE1A
30   and adenovirus made by directly amplifying
AdCMVlacZ viral stock. Specifically, the XhoI
digest produced visualized fragments of
approximately 1.4, 2.4, 2.5, 4.3, 5.0, and 15-20
kb; and the SalI digest produced fragments of
35   approximately 25 and 7 kb. This analysis showed
that the starting AdCMVlacZ virus, and that
produced by the infection of PC-3 cells in the

presence of pE1A, yielded similar fragments demonstrating that the cotransduction of pE1A and AdCMVlacZ resulted in the production of new, replication defective adenovirus, AdCMVlacZ.

A similar analysis of the virus made by cells cotransduced with AdCMVluc and pE1A also showed the new virus was identical to the starting AdCMVluc virus, as reported in Goldsmith 1994 Human Gene Ther. 5:1341.

    e.   <u>Demonstration of New Adenovirus Production by Cells Cotransduced with Viral and Plasmid DNAs</u>

Experimentation established that cells cotransduced with both replication-enabling plasmid and recombinant adenoviral DNA led to the production of recombinant adenovirus. This approach has utility in that it is possible to use viral-free methods for the initial introduction of the nucleic acids into cells which subsequently can locally produce the recombinant adenovirus *in vivo*. Viral free systems may have advantages with respect to cost and safety over viral systems, but suffer from lower efficiency of gene transfer. The ability to use viral-free approaches to administer the viral nucleic acids so that recombinant virus could be locally made *in vivo* capitalizes on the advantages of both viral-free and viral gene transfer systems.

AdCMVluc viral DNA was prepared as described previously. The AdCMVluc viral DNA and pE1A plasmid DNA were cotransduced into HeLa cells using cationic lipid, 1,2-dimyristoyl-3-trimethylammonium-propane (DOTAP), as a facilitator of DNA transfection. Experimentation has established that the molar ratio of replication-enabling plasmid DNA: viral DNA is a critical variable, as is the ratio of DOTAP to total DNA being introduced to the cells. These experiments

43

have shown that a molar ratio (plasmid:viral) of
approximately 1:1 to 4:1 is necessary in
conjunction with a lipid to DNA ratio of 2:1.
Unlike the original method of codelivery (using
5   plasmid ionically linked to the virus exterior)
where new virus production was evident at 72 hrs,
no new virus could be detected until 6-8 days post-
transduction of the DNAs.

The plasmid and viral DNAs were gently mixed
10 together in OPTIMUM® (GIBCO/BRL) to produce a total
DNA concentration of 100 ng/μl. In a separate
tube, DOTAP was mixed with OPTIMUM® to yield a
lipid concentration of 200 ng/μl. The DNA tube
contents were then added and gently mixed with the
15 DOTAP tube, and the tube incubated at room
temperature for 10 minutes. HeLa cells (10^5
cells/well of a 24 well plate) were washed, and 200
μl of OPTIMUM® (GIBCO/BRL) added. After the
DOTAP/DNA incubation period, 10 μl of the mixture
20 was added to each well of the HeLa's that were
subsequently placed in the usual 37OC humidified/CO_2
atmosphere. Four hrs later, the media was
aspirated and replaced with DMEM/F12 supplemented
with 2% fetal calf serum media. Supernatants were
25 collected 2, 4, 6, and 8 days later and examined
for the capacity to transfer luciferase activity to
fresh HeLa cells as evidence of new AdCMVluc
production. Luciferase activity was only conferred
by supernatants from the cells 6-8 days post-
30 transduction as graphically depicted in Figure 4.

    f.   Demonstration that Replication-Enablement
        Increases Gene Transfer in Tumor Tissue
        in vivo

Experiments suggest that the replication-
35 enablement technology amplifies adenoviral-
delivered transgene expression within a prostate
carcinoma model system. The system was tested by
measuring the amount of adenoviral transgene

expression, i.e., luciferase activity, within
tumors engrafted in nude mice. Tumors were produced
by administering the PC-3 cells (1 x 10⁷ cells SQ,
flanks) that achieved an 8-10 mm diameter in

5   approximately four weeks in athymic nude mice. At
that time, each tumor was injected once with 25 μl ·
of AdCMVluc/pE1A complex made as previously
described using a 27 ga. needle, so that each tumor
received 8.3 x 10⁸ viral particles complexed with

10   198 ng plasmid DNA. The tumors were excised 10
days later, and lysates made with a polytron on
ice, from which luciferase activities and total
protein concentrations were measured as described
above. In the tumors that were evaluated, this

15   analysis showed that those cotransduced with AdCMV-
luc and pUC 13 (n=7) had significantly lower
luciferase activity per mg of protein compared to
tumors cotransduced with the same virus and pE1A
(n=8) (Figure 3). Other experiments have shown a

20   consistently increased amount of luciferase
expression in the tumors that received the virus
and the replication-enabling plasmid compared to
controls receiving virus and pUC 13 as graphically
depicted in Figure 5, although significant

25   variability is present within the groups. These
results show that replication-enablement of the
AdCMV-luc virus within a tumor mass led to greater
transfer of the adenoviral transgene.

       **Example 2.   Creation of Syngeneic Recombinant
30   Virus-Producing Cells by
                         Cotransduction of Trans-
                         Complementing Plasmid Constructs.**

       Recombinant retrovirus is conventionally made
by passaging a retroviral vector through a

35   "packaging cell line" that has been stably
transfected with complementing viral protein coding
genes. The sequences of the vector and the
packaging sequences are designed so that only the

45

recombinant viral genome will be packaged into new
viral particles. The result is an infectious
particle that cannot replicate new virus. One
widely used retroviral packaging cell line is PA317
5   that was made by transfecting mouse fibroblasts
with the pPAM3 plasmid (Miller 1986).

The appropriate plasmids were cotransduced as
follows in order to demonstrate that a plasmid
containing the genes required for replication
10   obtained from a packaging cell line and retroviral
vector could be co-delivered with the resulting
production of new recombinant retrovirus. The
pPAM3 plasmid is described by Miller 1986, as noted
above. A representative retroviral vector, pBAG,
15   that contained a neomycin resistance gene as a
selectable marker and a bacterial lac Z gene as
described by Price 1987, was obtained from C.Cepko
(Harvard University, Cambridge, MA). Figure 6A is
a map of pBAG. Figure 6B is a schematic of
20   retroviral sequences within the pPAM3 plasmid.

The plasmids were introduced into cells by
ionic attachment to the exterior of an adenovirus.
The human type 5 adenovirus, DL1014 (Bridge 1989),
was modified to contain polylysine on the exterior
25   using the techniques described in Example 1. The
pPAM3 and pBAG plasmids were mixed in varying
proportions: 1 μg pBAG + 5 μg pPAM3, 3 μg pBAG + 3
μg pPAM3, 5 μg pBAG + 1 μg pPAM3, and 6 μg pBAG
alone, in a total volume of 250 μl HBS and added to
30   2.5 X 10^{10} polylysine-conjugated viral particles.
After incubation at room temperature for 30 min,
free polylysine was added as described in Example
1. A549 cells or PC-3 cells, both at densities of
1.5-2.0 X 10^{5}/35 mm plate, received 1/10 of each
35   complex in triplicate to quadruplicate for 2 hrs,
after which the media was changed to regular medium
and the cells grown under usual conditions. Three

days post-transduction with the two plasmids, supernatant was aspirated from the cells, clarified by centrifugation followed by 0.45 μM filtration and frozen at -70°C until further testing.

5   The presence of recombinant retrovirus in the supernatant was demonstrated by standard titer assays. Briefly, the supernatant was diluted by log 10 in regular media supplemented with polybrene at 5 μg/ml. The diluted supernatant was applied to

10   A549 cells (5 X 10⁵ cells/60 mm plate) overnight, after which the media was changed to regular media. One day following the media change, the A549 cells were enzymatically detached with trypsin and 1/10 of the cells plated in a fresh culture dish in

15   media supplemented with G418 1 mg/ml. Only cells containing the neomycin resistance gene supplied by the recombinant retrovirus can survive in the G418 media. The result after 10 to 14 days was the appearance of G418-resistant colonies of cells,

20   each colony representing the progeny of a single resistant cell. Figure 5 is a graphic representation of the titers of retrovirus in supernatants of cells cotransduced with pBAG and pPAM3. Supernatants were collected 72 hrs post-

25   transduction with the complementary retroviral vectors. In the supernatant obtained from the cotransduced A549 cells, titers were highest in the groups that received 1 μg pBAG + 5 μg pPAM3 at 4 X 10²/ml. A similar pattern was observed in PC-3

30   cells that were cotransduced with varying proportions of the complementing plasmids, resulting in titers of 1 X 10³/ml.

       As further evidence that new recombinant retrovirus had been made, resistant colonies were

35   shown to express the lac Z gene product. The colonies were washed thrice with phosphate buffered saline, fixed 10 minutes at 4°C in 0.2%

47

glutaraldehyde, 50 mM sodium phosphate buffer pH
7.3.   Following the fixation period, the plates
were washed thrice with phosphate buffered saline,
then incubated with the lac Z protein substrate, 5-

5   bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-
Gal) in "X-Gal Solution" (80 mM Na_2HPO_4, 20 mM
NaH_2PO_4, 1.3 mM $MgCl_2$, 1 mg/ml X-Gal, 3 mM $K_3Fe(CN)_6$,
3 mM $K_4Fe(CN)_6$) at 37°C overnight.   Cells containing
the lac Z protein turn blue after this treatment.

10   Most of the colonies were stained deeply blue,
indicating the presence of the lac Z product
provided by the recombinant retrovirus.

We claim:

1.   A method for creating recombinant virus-producing cells comprising

selecting a first nucleic acid sequence comprising a viral genome which is not capable of directing the production of new viral particles in the absence of additional viral nucleic acid sequence,

constructing a second nucleic acid sequence comprising a viral gene sequence consisting essentially of the viral genes required for the first nucleic acid sequence to produce new viral particles in a cell in which the first nucleic acid sequence is otherwise unable to direct the production of new viral particles, in combination with means for replicating the second nucleic acid sequence, and

inserting the combination of the first and second nucleic acid sequences into the cell in which the first nucleic acid sequence is unable to direct the production of new viral particles in the absence of the second nucleic acid sequence.

2.   The method of claim 1 wherein the first and second nucleic acid sequences are coupled.

3.   The method of claim 2 wherein the sequences are coupled covalently.

4.   The method of claim 2 wherein the sequences are coupled ionically.

5.   The method of claim 1 wherein the first and second nucleic acid sequences are inserted by transduction.

6.   The method of claim 1 wherein the first and second nucleic acid sequences are inserted by means of a virus capable of infecting the cell.

7.   The method of claim 6 wherein the virus is rendered non-viable prior to infection.

8.   The method of claim 1 wherein the first or
second nucleic acid sequences further comprise
nucleic acid sequence selected from the group
consisting of nucleic acid sequence that codes for
5   proteins used to identify cells infected with
recombinant virus produced by the combination of
the first and second nucleic acid sequences,
nucleic acid sequences that codes for proteins that
function to kill cells containing the viral genome,
10   nucleic acid sequence that codes for therapeutic
proteins that will serve to treat a
pathophysiologic condition within the body, and
nucleic acid sequence encoding biologically active
nucleic acids.

15   9.   The method of claim 8 wherein the first
nucleic acid sequences comprising coding nucleic
acid sequence.

         10. The method of claim 1 wherein the virus
is selected from the group consisting of
20   adenoviruses, herpesviruses, adeno-associated
viruses, and retroviruses.

         11. A composition for creating recombinant
virus-producing cells comprising
         a first nucleic acid sequence comprising a
25   viral genome which is not capable of directing the
production of new viral particles and
         a second nucleic acid sequence comprising
             a viral gene sequence consisting
essentially of the genes required for the first
30   nucleic acid sequence to direct the production of
new viral particles in a cell in which the first
nucleic acid sequence is otherwise unable to direct
the production of new viral particles
             in combination with means for replicating
35   the second nucleic acid sequence in a cell.

         12. The composition of claim 11 further
comprising a cell in which the first nucleic acid

sequence is unable to direct the production of new
viral particles in the absence of the combination
of the first and second nucleic acid sequences.

13. The composition of claim 11 wherein the
first and second nucleic acid sequences are
coupled.

14. The composition of claim 13 wherein the
sequences are coupled covalently.

15. The composition of claim 13 wherein the
sequences are coupled ionically.

16. The composition of claim 11 further
comprising an agent facilitating transduction of a
mammalian cell by the first and second nucleic acid
sequences.

17. The composition of claim 11 wherein the
first or second nucleic acid sequences further
comprise nucleic acid sequence selected from the
group consisting of nucleic acid sequence that
codes for proteins used to identify cells infected
with recombinant virus produced by the combination
of the first and second nucleic acid sequences,
nucleic acid sequences that codes for proteins that
function to kill cells containing the viral genome,
nucleic acid sequence that codes for therapeutic
proteins that will serve to treat a
pathophysiologic condition within the body, and
nucleic acid sequence encoding biologically active
nucleic acids.

51

18.   A method for expressing exogenous nucleic
acid sequence in a mammalian cell comprising
introducing into a cell
        a first nucleic acid sequence comprising a
5   viral genome which is not capable of directing the
production of new viral particles and
        a second nucleic acid sequence comprising
            a viral gene sequence consisting
essentially of the genes required for the first
10   nucleic acid sequence to direct the production of
new viral particles in a cell in which the first
·   nucleic acid sequence is unable to otherwise direct
the production of new viral particles
            in combination with means for replicating
15   the second nucleic acid sequence in a cell,
        wherein the first or second nucleic acid
sequence is selected from the group of nucleic acid
sequence consisting of nucleic acid sequence that
codes for proteins used to identify cells infected
20   with recombinant virus produced by the combination
of the first and second nucleic acid sequences,
nucleic acid sequences that codes for proteins that
function to kill cells containing the viral genome,
nucleic acid sequence that codes for therapeutic
25   proteins that will serve to treat a
pathophysiologic condition within the body, and
nucleic acid sequence encoding biologically active
nucleic acids.
        19.   The method of claim 18 wherein the first
30   and second nucleic acid sequences are coupled.
        20.   The method of claim 19 wherein the
sequences are coupled covalently.
        21.   The method of claim 19 wherein the
sequences are coupled ionically.
35   22.   The method of claim 18 wherein the cells
are in culture.

23. The method of claim 18 wherein the cells are in a patient.

24. A method for killing replicating cells comprising

administering to the replicating cells an adenovirus which is unable to direct the production of new viral particles in the replicating cells,

administering to the replicating cells a retrovirus comprising

a viral gene sequence consisting essentially of the genes required for the adenovirus to direct the production of new viral particles in the replicating cells in which the adenovirus is otherwise unable to direct the production of new viral particles.

25. The method of claim 24 wherein the replicating cells are tumor cells.

26. The method of claim 25 wherein the replicating cells are in the brain.

27. A composition for killing replicating cells comprising

an adenovirus which is unable to direct the production of new viral particles in the replicating cells, and

a retrovirus comprising

a viral gene sequence consisting essentially of the genes required for the adenovirus to direct the production of new viral particles in the replicating cells in which the adenovirus is otherwise unable to direct the production of new viral particles.

28. The composition of claim 27 wherein the replicating cells are tumor cells, further comprising the cells.
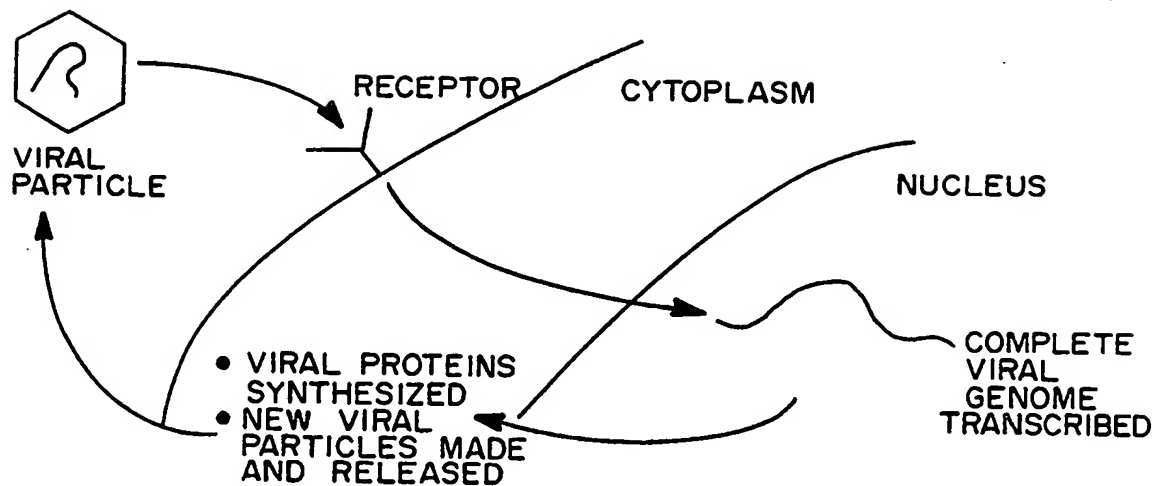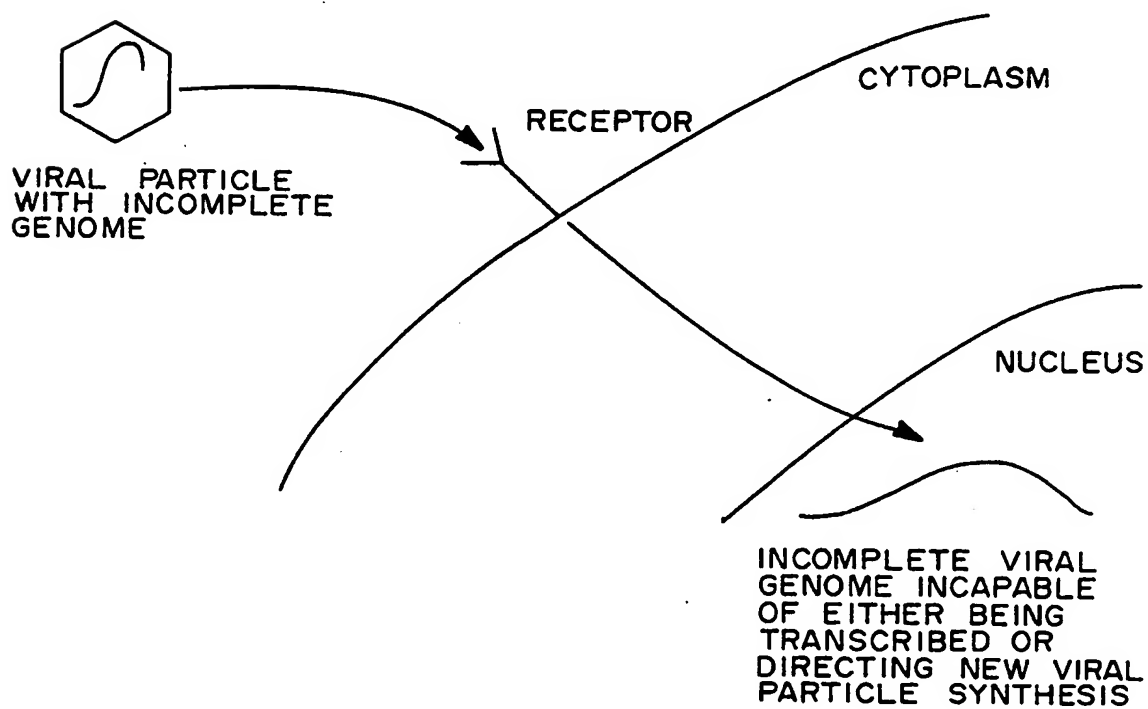
FIG. 1a

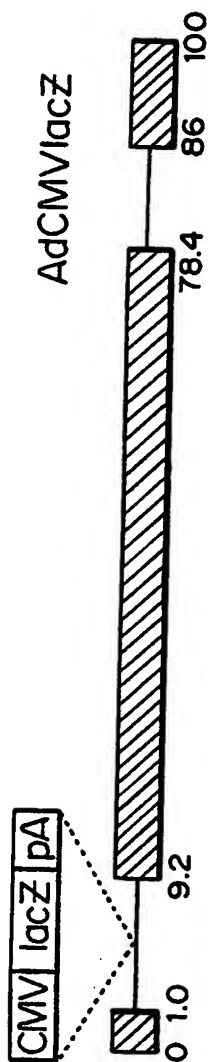RECEPTOR CYTOPLASM
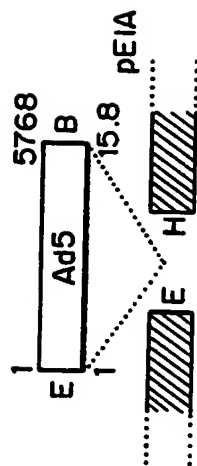
VIRAL PARTICLE

NUCLEUS

• VIRAL PROTEINS SYNTHESIZED
• NEW VIRAL PARTICLES MADE AND RELEASED

COMPLETE VIRAL GENOME TRANSCRIBED

FIG. 1b

CYTOPLASM

RECEPTOR

VIRAL PARTICLE WITH INCOMPLETE GENOME

NUCLEUS

INCOMPLETE VIRAL GENOME INCAPABLE OF EITHER BEING TRANSCRIBED OR DIRECTING NEW VIRAL PARTICLE SYNTHESIS

FIG. 2a

AdCMVlacZ

FIG. 2b
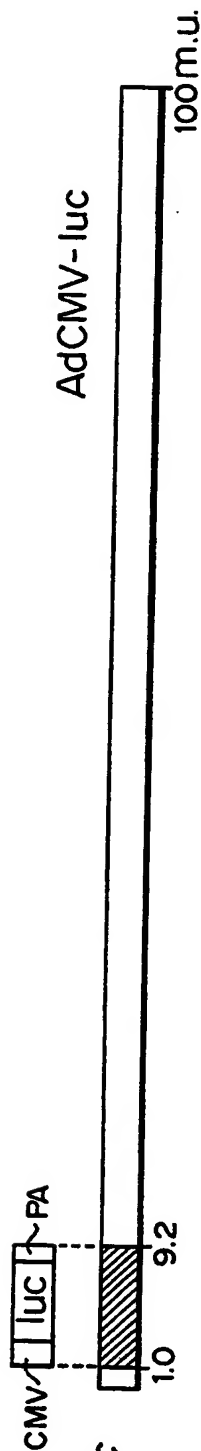
FIG. 2c

AdCMV-luc
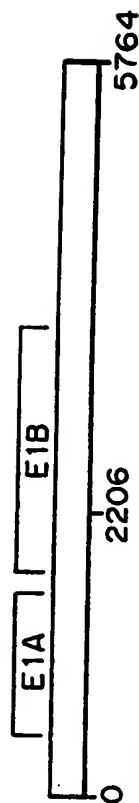
FIG. 2d

Adenovirus sequences 1-5764 used in pE1A. Adenovirus sequences 1-2206 used in pE2206.

FIG. 3

plasmid cotransduced with AdCMVluc DNA

FIG. 4

FIG. 5

FIG. 6a

MoIMLV DNA: ▭

mouse genomic DNA: ▥

mRNA start sites: ↑

3) ALL RECOGNITION SITES FOR THE LISTED RESTRICTION ENZYMES ARE SHOWN.

4) REFERENCE: PNAS 84:156-160, 1987

9TH AMINO ACID OF WILD TYPE β-gal GENE

...ATG. GGC GCG GAT CCC GTC...
met gly ala asp pro val

BamHI(1074)

TOTAL PLASMID SIZE- 12,220bp

NOTES: 1)THERE IS NO AMP GENE IN THIS PLASMID. THE NEO GENE CAN BE SELECTED IN BACTERIA BY GROWTH IN 50 μg/ml KANAMYCIN SULFATE.

2)THE ATG FOR THE β-gal GENE (ORIGINALLY THE ATG FOR THE VIRAL pr65 gag PROTEIN) IS LOCATED 5' TO THE BamHI SITE AT 1074:

FIG. 6b

FIG. 7